BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

As rescanning documents *will not* correct images, please do not report the images to the Image Problem Mailbox.

Carbon Monoxide Generated by Heme Oxygenase-1 Suppresses the Rejection of Mouse-to-Rat Cardiac Transplants[1]

Koichiro Sato,[2]* Jozsef Balla,† Leo Otterbein,‡ R. Neal Smith,§ Sophie Brouard,* Yuan Lin,* Eva Csizmadia,* Jean Sevigny,* Simon C. Robson,* Gregory Vercellotti,¶ Augustine M. Choi,‡ Fritz H. Bach,[3]* and Miguel P. Soares[4]*

Mouse-to-rat cardiac transplants survive long term after transient complement depletion by cobra venom factor and T cell immunosuppression by cyclosporin A. Expression of heme oxygenase-1 (HO-1) by the graft vasculature is critical to achieve graft survival. In the present study, we asked whether this protective effect was attributable to the generation of one of the catabolic products of HO-1, carbon monoxide (CO). Our present data suggests that this is the case. Under the same immunosuppressive regimen that allows mouse-to-rat cardiac transplants to survive long term (i.e., cobra venom factor plus cyclosporin A), inhibition of HO-1 activity by tin protoporphyrin, caused graft rejection in 3–7 days. Rejection was associated with widespread platelet sequestration, thrombosis of coronary arterioles, myocardial infarction, and apoptosis of endothelial cells as well as cardiac myocytes. Under inhibition of HO-1 activity by tin protoporphyrin, exogenous CO suppressed graft rejection and restored long-term graft survival. This effect of CO was associated with inhibition of platelet aggregation, thrombosis, myocardial infarction, and apoptosis. We also found that expression of HO-1 by endothelial cells in vitro inhibits platelet aggregation and protects endothelial cells from apoptosis. Both these actions of HO-1 are mediated through the generation of CO. These data suggests that HO-1 suppresses the rejection of mouse-to-rat cardiac transplants through a mechanism that involves the generation of CO. Presumably CO suppresses graft rejection by inhibiting platelet aggregation that facilitates vascular thrombosis and myocardial infarction. Additional mechanisms by which CO overcomes graft rejection may involve its ability to suppress endothelial cell apoptosis. *The Journal of Immunology,* **2001, 166: 4185–4194.**

Survival of a transplanted organ is thought to relate mainly to the success of immunosuppression, in terms of blocking the immune response that leads to graft rejection. However, it is becoming clear that the graft itself can mitigate the pathological consequences of this immune response and therefore contribute to promote its own survival (1–3). We have investigated this phenomenon in mouse hearts transplanted into rats, a model of xenotransplantation (1, 4–6). In this experimental situation, graft rejection occurs 2–3 days after transplantation, through a process referred to as acute vascular rejection (4–7). This type of rejection can occur in the absence of T cells, through the generation of T cell-independent Abs of the IgM isotype (8). Complement activation by these Abs leads to endothelial cell activation, which is associated with the expression of a series of proinflammatory genes that promote thrombosis as well as activation of host leukocytes. Presumably, expression of these proinflammatory genes contributes in a critical manner to the pathogenesis of acute vascular rejection (9).

Rejection of mouse-to-rat cardiac transplants is prevented when the recipient is treated at the time of transplantation with cobra venom factor (CVF)[5] to block complement activation and daily thereafter with cyclosporin A (CsA) to block T cell activation (4). We refer to graft survival under this regimen as "accommodation" (5, 10). Naive grafts transplanted into the same recipient 10 days after transplantation of the first graft undergo hyperacute rejection in a few minutes, suggesting that accommodated grafts are actively protected against rejection (5, 11). We have argued that accommodation is dependent on the expression by the graft vasculature of a series of protective genes (3, 5) including the stress-responsive gene heme oxygenase-1 (HO-1; Refs. 1, 3–6, 12). We have shown that this is the case, and in particular that the expression of the protective gene HO-1 contributes in a critical manner to establish accommodation. This is illustrated by the observation that hearts

*Immunobiology Research Center, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA 02215; †Department of Medicine, University Medical School of Debrecen, Hungary; ‡Yale University, School of Medicine, Department of Internal Medicine, Pulmonary and Critical Care Section, New Haven, CT 06520; §Department of Pathology, Massachusetts General Hospital, Boston, MA 02114; and ¶Division of Hematology, University of Minnesota, Minneapolis, MN 55455

Received for publication October 16, 2000. Accepted for publication January 4, 2001.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by Grant Roche Organ Transplantation Reserch Foundation-998521355 (awarded to M.P.S.) and National Institutes of Health Grant HL58688 (awarded to F.H.B.). M.P.S. is a Phyllis and Paul Fireman 2000 fellow at the Beth Israel Deaconess Medical Center, Harvard Medical School. S.B. was partially supported by a grant from Association pour la Recherche sur le Cancer and by a grant from Institut National de la Santé et de la Recherche Médicale. This work was partially supported by Novartis Pharma, Basel, Switzerland.

[2] Current address: Second Department of Surgery, Tohoku University Hospital, Sendai, Japan.

[3] F.H.B. is the Lewis Thomas Professor at the Harvard Medical School and is a paid consultant for Novartis Pharma.

[4] Address correspondence and reprint requests to Dr. Miguel P. Soares, Immunobiology Research Center, Beth Israel Deaconess Medical Center, Harvard Medical School, 99 Brookline Avenue, Boston, MA 02215. E-mail address: msoares@caregroup.harvard.edu

[5] Abbreviations used in this paper: CVF, cobra venom factor; CsA, cyclosporin A; HO, heme oxygenase; CO, carbon monoxide; Mϕ, macrophages; CoPPIX, cobalt protoporphyrin; FePPIX, iron protoporphyrin; SnPPIX, tin protoporphyrin; Act.D, actinomycin D; ppm, parts per million; H&E, hematoxylin and eosin; CH50, complement hemolytic assay.

0022-1767/01/$02.00

from HO-1-deficient (HO-$1^{-/-}$) mice transplanted into rats treated with CVF plus CsA undergo acute vascular rejection, whereas hearts from wild-type HO-$1^{+/+}$ mice transplanted under the same regimen accommodate and survive long term (1). The mechanism by which HO-1 promotes accommodation remains to be established.

HOs are the rate-limiting enzymes in the catabolism of heme into bilirubin, free iron, and carbon monoxide (CO; Refs. 13, 14). Expression of HO-1 is up-regulated in most cell types exposed to oxidative stress, whereas the constitutive expression of HO-2 is not up-regulated under these conditions (13, 14). Analyses of HO-$1^{-/-}$ mice suggest that HO-1 regulates iron homeostasis (15) while acting as a cytoprotective gene (16). In addition, HO-1 may have potent antiinflammatory (16–19) and antiapoptotic effects (1, 20, 21). Findings consistent with such biological functions were confirmed in a case report of HO-1 deficiency in humans (22).

The molecular mechanism(s) responsible for the cytoprotective effects of HO-1 remain largely unknown. The current view is that HO-1 has a diverse spectrum of cytoprotective effects that are associated with the different end products of heme catabolism (13, 14). One these products, i.e., CO, has potent cytoprotective effects (23). These include the induction of vasorelaxation and suppression of platelet aggregation (24–26), both of which are mediated through the activation of the enzyme guanylyl cyclase and subsequent generation of cGMP. In addition, CO inhibits the proinflammatory phenotype associated with the activation of monocyte/macrophages (Mϕ; Ref. 27). CO also protects endothelial cells from undergoing apoptosis (1, 28). Both these biological actions of CO act through the activation of the p38 mitogen-activated protein kinase pathway, independently of the activation of guanylyl cyclase or cGMP generation (27, 28). Presumably, these biological properties of CO contribute in a critical manner to the overall cytoprotective and antiinflammatory actions of HO-1 and thus may be a central component on the mechanism by which HO-1 suppresses the rejection of transplanted organs (1, 29, 30). In this study, we tested whether or not the ability of HO-1 to suppress the rejection of mouse-to-rat cardiac grafts depends on the generation of CO. Our results suggest that this is the case.

Materials and Methods

Animals

BALB/c mouse hearts were used as donor organs for transplantation into inbred adult male Lewis rats (Harlan Sprague-Dawley, Indianapolis, IN). Animals were housed in accordance with guidelines from the American Association for Laboratory Animal Care, and research protocols were approved by the Institutional Animal Care and Use Committees of the Beth Israel Deaconess Medical Center.

Surgical model

Animals were anesthetized by a combination of methoxyflurane (Pitman-Moore, Mundelain, IL) inhalation and pentobarbital (Abbott, North Chicago, IL) at a dose of 30–50 mg/kg i.p. during all procedures. Heterotopic cardiac transplants were performed as described before (1, 4). Graft survival was assessed daily by palpation, and rejection was diagnosed by cessation of ventricular contractions and confirmed by histologic examination.

Experimental reagents

CVF (Quidel, San Diego, CA) was administered i.p. on day −1 (60 U/kg) and on day 0 (20 U/kg) with respect to the day of transplantation (day 0). CsA (Novartis Pharma, Basel, Switzerland) was administered daily i.m. (15 mg/kg) starting at day 0 and daily thereafter until the end of each experiment. Tin protoporphyrin (SnPPIX), cobalt protoporphyrin (CoPPIX), and iron protoporphyrin (FePPIX; Porphyrin Products, Logan, UT) were diluted in 100 mM NaOH to a stock solution of 50 mM and kept at −70°C until used. Light exposure was limited has much as possible. Both SnPPIX and FePPIX were administered i.p. (30 μM/kg) in PBS. FePPIX and SnPPIX were administered to the donor at days −2 and −1 (30 μM/kg) and to the recipient at the time of transplantation (day 0) and daily thereafter (30 μM/kg).

CO exposure

Briefly, CO at a concentration of 1% (10,000 parts per million; ppm) in compressed air was mixed with balanced air (21% oxygen) in a stainless steel mixing cylinder before entering the exposure chamber. CO concentrations were controlled by varying the flow rates of CO in a mixing cylinder before delivery to the chamber. Because the flow rate is primarily determined by the O_2 flow, only the CO flow was changed to deliver the final concentration to the exposure chamber. A CO analyzer (Interscan Corporation, Chatsworth, CA) was used to measure CO levels continuously in the chamber. Graft donors were placed in the CO exposure chamber 2 days before transplantation. Graft recipients were placed in the exposure chamber immediately following transplantation and were kept in the exposure chamber during 14 (n = 3) or 16 (n = 3) days. CO concentration was maintained between 250 and 400 ppm at all times. Animals were removed daily from the chamber to assess for graft survival and to administer CsA, SnPPIX, or FePPIX, as described above.

HO enzymatic activity

HO enzymatic activity was measured by bilirubin generation in heart and liver microsomes. Animals were sacrificed, and the liver and hearts were flushed with ice-cold PBS and frozen at −70°C until used. Organs were homogenized in four volumes of sucrose (250 mM) Tris-HCl (10 mM/L) buffer (pH 7.4) on ice and centrifuged (28,000 × g, 20 min, 4°C). The supernatant was centrifuged (105,000 × g, 60 min, 4°C), and the microsomal pellet was resuspended in $MgCl_2$ (2 mM/L)-potassium phosphate (100 mM) buffer (pH 7.4) and sonicated on ice. The samples (1 mg of protein) were added to the reaction mixture (400 μl) containing rat liver cytosol (2 mg of protein), hemin (50 μM), glucose-6-phosphate (2 mM), glucose-6-phosphate dehydrogenase (0.25 U), and NADPH (0.8 mM) for 60 min at 37°C in the dark. The formed bilirubin was extracted with chloroform and Δ OD was measured at 464–530 nm (extinction coefficient, 40 mM/L/cm for bilirubin). Enzyme activity is expressed as picomoles of bilirubin formed per milligram of protein per 60 min (pmol/mg/h). The protein concentration was determined by the bicinchoninic acid protein assay (Pierce, Georgetown). The background of the technique was ~5 pmol/mg/h. All reagents used in this assay were purchased from Sigma (St. Louis, MO), unless otherwise indicated.

Carboxyhemoglobin was measured 2 days after transplantation by using a Corning 865 blood gas analyzer (Clinical Chemistry, Massachusetts General Hospital, Boston, MA).

Histomorphometric analysis

Grafts were harvested 3 days after transplantation, embedded in paraffin, fixed in formalin, and serially sectioned (5 μm) in toto from the apex to the base. Ten sections were placed per slide in a total of about 20–25 slides. Every fifth slide was stained with hematoxylin and eosin (H&E) for histomorphometric analysis. Two images per slide were captured by using a Nikon Eclipse E600 microscope (Nikon, Melville, NY) connected to a Hitachi 3-CCD Color Camera (model HV-C20; Hitachi, Tokyo, Japan) and to a Power Macintosh 7300/200 computer (Apple Computer, Cupertino, CA) equipped with IPLab Spectrum digital imaging software (Signal Analytics Corporation, Vienna, VA). About 50 images were captured from each transplanted heart from two to three animals per group. Images were analyzed by manual segmentation, tracing the infarcted and noninfarcted areas from the right and left ventricles in each section. Areas corresponding to infarcted and noninfarcted tissue were calculated by digital imaging software as number of pixels corresponding to those areas. Infarcted and noninfarcted areas were then calculated as percentage of total area. Pooled data for each group, expressed as area in pixels or as percentage of infarction, was analyzed by using ANOVA. Results obtained in this manner were similar whether using either pixels or percentage of infarction and only the results obtained using percentage of infarction are shown (see Table 3). Results are expressed as mean ± SD.

Immunohistology

Grafts were harvested 3 days after transplantation, snap-frozen in liquid nitrogen, and stored at −80°C. Cryostat sections were fixed and stained as described previously (1). Rat leukocyte populations were analyzed by using anti-rat leukocyte common Ag (LCA, CD45; OX-1), $\alpha\beta$ TCR (TCR$\alpha\beta$-chains; R73), B cell (CD45RB; OX-33), NK cell (NKR-P1; 3.2.3), Mϕ (CD68; ED-1), and mAbs (Serotec, Harlan Bioproducts for Science, Indianapolis, IN). Detection of fibrin/fibrinogen was conducted by

Table I. *Inhibition of HO-1 activity by SnPPIX precipitates graft rejection*[a]

Treatment	Survival Time (days)
CVF + CsA	>50 (n = 8)
CVF + CsA + FePPIX	>50 (n = 4)
CVF + CsA + SnPPIX	3, 4, 5 (n = 2); 6 (n = 4); 7 (n = 2)

[a] Mouse hearts were transplanted into CVF plus CsA-treated rats. When indicated, graft recipients were treated with FePPIX or SnPPIX, as described in *Materials and Methods*. Treatment with SnPPIX induced graft rejection 3–7 days after transplantation ($p < 0.0001$ as compared to rats treated with CVF plus CsA alone or with CVF plus CsA plus FePPIX). Statistical analyzes were carried out using Fisher's exact test.

using a rabbit anti-human fibrin/fibrinogen polyclonal Ab (Dako, Carpinteria, CA). Intragraft complement activation was detected by using an anti-rat C1q (The Binding Site, Birmigham, U.K.), C3 (ED11; Serotec), or C5b-9 mAbs (Dako). Rat IgM was detected by using the mouse anti-rat IgM mAb MARM-4 (a kind gift of Dr. H. Bazin, University of Louvain, Brussels, Belgium). Isotype-matched mAbs or purified Ig, as well as a control for residual endogenous peroxidase activity, were included in each experiment. Detection of apoptosis was conducted by using ApopTag in situ apoptosis detection kit (Oncor, Gaithersburg, MD) according to the manufacturer's instructions.

Complement hemolytic assay (CH50)

CH50 units were defined as the dilution of rat serum required to produce 50% maximal lysis of Ab-sensitized sheep erythrocytes. Briefly, Ab-sensitized sheep erythrocytes (1×10^8 cells/ml; Sigma) were incubated (30 min, 37°C) with rat serum in gelatin Veronal buffer (GVB^{++}; Sigma). Cells were centrifuged and hemoglobin release was measured (λ = 550 nm). Background was measured in the absence of sheep erythrocytes or in the absence of serum and subtracted from all samples.

Cellular ELISA

Serum levels of rat anti-mouse Abs were measured by cellular-based indirect ELISA. The mouse 2F-2B endothelial cell line (CRL-2168; American Type Culture Collection (ATCC), Manassas, VA) was used as an antigenic target. Briefly, 2F-2B cells were cultured in DMEM (Life Technologies, Rockville, MD), 10% FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin (Life Technologies). Glutharaldehyde-fixed 2F-2B cells were incubated (1 h, 37°C) in the presence of rat serum serially diluted in PBS 0.05% Tween 20 (Sigma) and rat anti-mouse Abs were detected by using mouse anti-rat IgM (MARM-4), IgG1 (MARG1-2), IgG2a (Marg2a-1), IgG2b (MARGb-8), or IgG2c (MARG2c-5) (kind gifts from Prof. H. Bazin, University of Louvain, Brussels, Belgium). Mouse anti-rat Abs were detected by using HRP-labeled goat anti-mouse Fab' depleted from anti-rat Ig cross reactivity (0.1 μg/ml, 1 h, room temperature; Pierce, Rockford, IL). HRP was revealed by using *ortho*-phenyldiamine (Sigma) and H_2O_2 (0.03%) in citrate buffer (pH 4.9). Absorbance was measured at λ = 490 nm. The relative amount of circulating anti-graft Abs in the serum was expressed as OD (λ = 490) taken from one serial dilution in the linear range of the assay (1:32–1:1024).

Binding of rat C3 to mouse endothelial cells was measured by a modified cellular ELISA with mouse 2F-2B endothelial cells as antigenic targets (8). Briefly, nonfixed 2F-2B endothelial cells were incubated in the presence of rat serum serially diluted in GVB^{++} buffer (1 h, 37°C). Cells were fixed in PBS, 0.05% glutharaldehyde, and rat C3 deposition was detected by using a mouse anti-rat C3 mAb (Serotec).

Platelet aggregation assay

Mouse 2F-2B endothelial cells were cultured on 0.2% gelatin (Sigma)-coated six-well plates in 88% DMEM (Life Technologies), 10% FCS (FCS), 100 U/ml penicillin, and 100 μg/ml streptomycin (Life Technologies). Confluent endothelial cells either were left untreated or were treated with the HO-inducing agent CoPPIX (50 μM; 18 h), the HO inhibitor SnPPIX (50 μM, 18 h), or both CoPPIX (50 μM, 15 h) and SnPPIX (50 μM, 3 h). Platelet-rich plasma was obtained by centrifugation ($290 \times g$, 12 min, 19°C) of normal rat plasma in 3.8% sodium citrate. Rat platelets (3×10^8 cells/ml) were resuspended in HT buffer (8.9 mM $NaHCO_3$, 0.8 mM KH_2PO, 5.6 mM dextrose, 2.8 mM KCl solution, 0.8 mM $MgCl_2$, 129 mM NaCl, 10 mM HEPES). Platelets were overlaid (5 min; 37°C) on mouse endothelial cells, and platelet aggregation assay were conducted as described before (31) by using an aggregometer (Chrono-Log, Harestown, PA) and ADP (0.5–4 μM) as an agonist.

Cell extracts and Western blot analysis

Endothelial cells were washed in PBS (pH 7.2), harvested by scraping, and lysed in Laemmli buffer. Electrophoresis was conducted under denaturing conditions with 10% polyacrylamide gels. Proteins were transferred onto a polyvinyldifluoridine membrane (Immobilon P; Millipore, Bedford, MA) by electroblotting and detected with rabbit polyclonal Abs directed against human HO-1 or HO-2 (StressGen, Victoria, Canada) or β-tubulin (Boehringer Mannheim, Mannheim, Germany). Proteins were visualized by using HRP-conjugated donkey anti-rabbit IgG or goat anti-mouse IgG (Pierce) and the ECL assay (Amersham Life Science, Arlington Heights, IL) according to manufacturer's instructions.

Transient transfections and apoptosis assay

The murine 2F-2B endothelial cell line (ATCC) was transiently transfected as described elsewhere (1, 28). All experiments were conducted 24–48 h after transfection. β-galactosidase-transfected cells were detected as described elsewhere (1, 28). Percentage of viable cells was assessed by evaluating the number of β-galactosidase-expressing cells that retained normal morphology as described elsewhere (1, 28). The number of random fields counted was determined to have a minimal of 200 viable transfected cells per control well. The percentage of viable cells was normalized for each DNA preparation to the number of transfected cells counted in the absence of the apoptosis-inducing agent (100% viability). All experiments were performed at least three times in duplicate. Actinomycin D (Act.D; Sigma) was dissolved in PBS and added to the culture medium (10 μg/ml) 24 h after transfection. SnPPIX (Porphyrin Products) was dissolved (10 mM) in 100 mM NaOH and conserved at −20°C until used. SnPPIX was added to the culture medium (50 μM) 6 h after transfection. Human recombinant

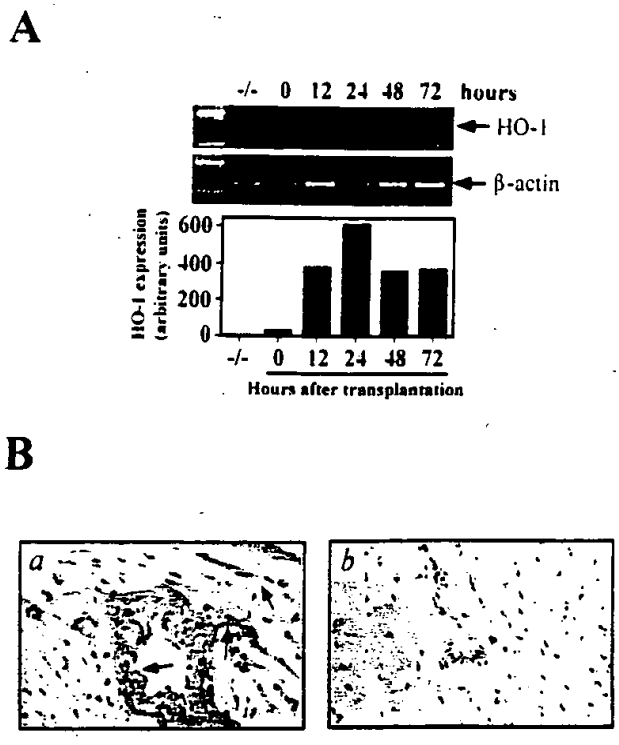

FIGURE 1. Expression of HO-1 in mouse hearts transplanted into CVF plus CsA-treated rats. Mouse hearts were transplanted into rats treated with CVF plus CsA. *A*, Expression of HO-1 and β-actin mRNA were detected by RT-PCR. −/− indicates RNA from HO-$1^{-/-}$ mouse hearts used as a negative control. Histograms represent relative level of HO-1 mRNA expression normalized for expression of β-actin mRNA. *B*, Mouse hearts were harvested 3 days after transplantation into CVF plus CsA-treated rats. *a*, HO-1 Expression was detected using an anti-HO-1 polyclonal rabbit anti-serum. Positive staining is indicated by black arrows. *b*, Negative control was conducted by using nonspecific anti-serum. Stainings shown are representative of at least five samples analyzed.

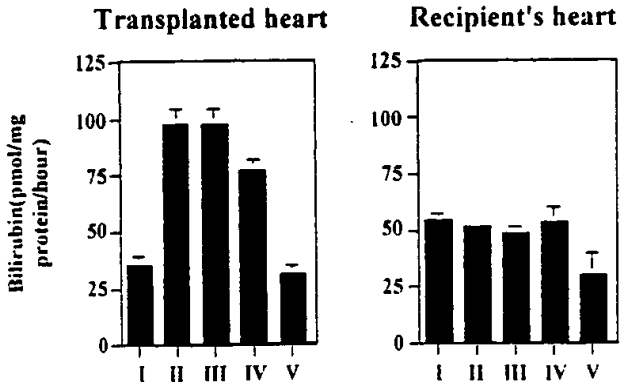

FIGURE 2. SnPPIX Inhibits HO-1 enzymatic activity in vivo. *A*, Mouse hearts were transplanted into untreated rats (II) or into rats treated with, CVF and CsA (III) plus FePPIX (IV) or SnPPIX (V). Total HO activity in donor and recipient hearts was measured 2 days after transplantation and compared with basal HO activity in normal mouse and rat hearts, respectively (I). Results shown are the mean ± SD of three animals analyzed for each treatment. Statistical analyses were conducted by using unpaired Welsh *t* test.

TNF-α (R&D Systems, Minneapolis, MN) was dissolved in PBS, 1% BSA and added to the culture medium (10–100 ng/ml) 24 h after transfection.

Exposure of cultured endothelial cells to CO

Cells were exposed to compressed air or varying concentration of CO (250 and 10,000 ppm), as described elsewhere (27, 28).

RT-PCR was conducted after RNA isolation from the transplanted hearts by using an RNA extracting kit, according with the manufacturer's instructions (Qiagen, Chatsworth, CA). Primers used for mouse β-actin were: sense, CCTGACCGAGCGTGGCTACAGC; antisense, AGCCTC CAGGGCATCGGAC; and for mouse HO-1: sense, TCCCAGACAC CGCTCCTCCAG; antisense, GGATTTGGGGCTGCTGGTTTC.

Results

HO-1 enzymatic activity is critical to suppress acute vascular rejection

Mouse hearts transplanted into untreated rats underwent acute vascular rejection 2–3 days after transplantation, an observation consistent with our previous reports (1, 4). Under CVF plus CsA treatment, mouse cardiac grafts survived long term (Table I), a finding also consistent with previous reports (1, 4). Under CVF plus CsA treatment, graft survival was associated with up-regulation of HO-1 expression by graft endothelial and smooth muscle cells as well as by cardiac myocytes (Fig. 1). Expression of HO-1 mRNA was detected 12–24 h after transplantation and HO-1 protein 24–72 h after transplantation (Fig. 1). Long-term graft survival did not occur when the HO inhibitor SnPPIX was administered to the donor and then to the recipient, despite treatment with CVF plus CsA. Under these conditions, all grafts were rejected in 3–7 days (Table I). Control treatment with FePPIX, a protoporphyrin that does not inhibit HO activity, did not lead to graft rejection (Table I).

To demonstrate that SnPPIX, but not FePPIX, blocked HO-1 function in vivo, total HO enzymatic activity was quantified in transplanted and recipient hearts 2 days after transplantation (Fig. 2). Naive mouse hearts produced 35.5 ± 4 picomols of bilirubin per milligram of total protein per hour (pmol/mg/h; Fig. 2). HO activity was significantly increased in mouse hearts transplanted into untreated (98 ± 7.21 pmol/mg/h; $p = 0.001$), CVF plus CsA (98.3 ± 7.23 pmol/mg/h)-treated, or CVF plus CsA plus FePPIX (77.3 ± 5.51 pmol/mg/h; $p = 0.0009$)-treated rats, as compared with naive hearts (Fig. 2). HO activity was inhibited to basal levels, as present in naive hearts, in mouse hearts transplanted into rats treated with CVF plus CsA plus SnPPIX (32.37 ± 7.23 pmol/mg/h). This represented a highly significant inhibition as compared with mouse hearts transplanted into untreated ($p = 0.0009$), CVF plus CsA ($p = 0.0009$)-treated, or CVF plus CsA plus FePPIX-treated rats ($p = 0.0018$; Fig. 2). HO activity in the recipient's

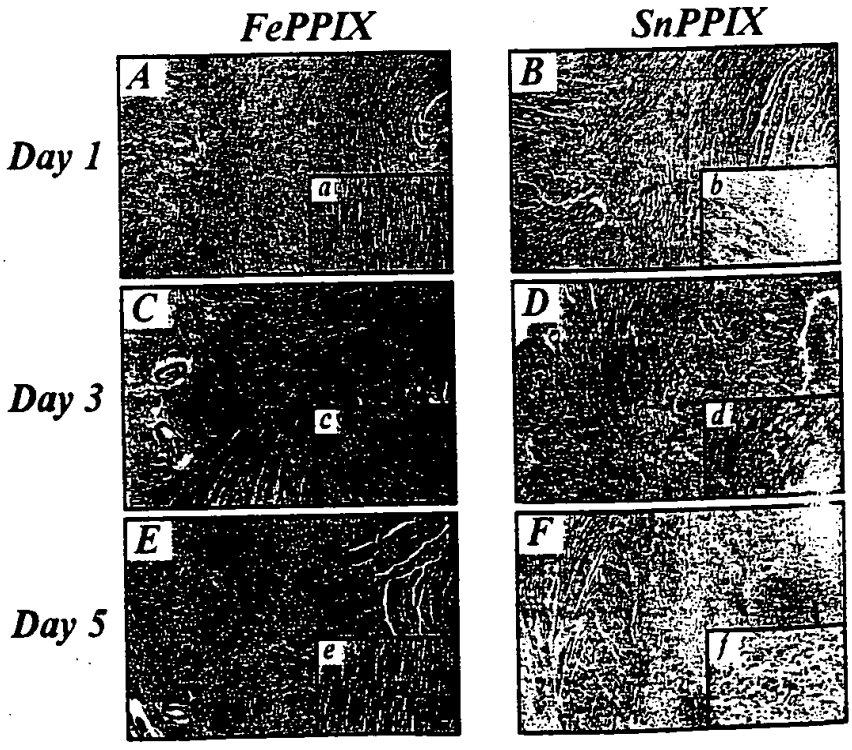

FIGURE 3. Mouse hearts transplanted into SnPPIX treated rats undergo myocardial infarction. Mouse hearts were transplanted into CVF plus CsA-treated rats that received FePPIX (*A*, *C*, and *E*) or SnPPIX (*B*, *D*, and *F*). Transplanted hearts were harvested 1 (*A* and *B*), 3 (*C* and *D*), or 5 (*E* and *F*) days after transplantation and stained with H&E. Samples *A–D* are ×80 magnifications and samples *a–d* ×600 magnifications.

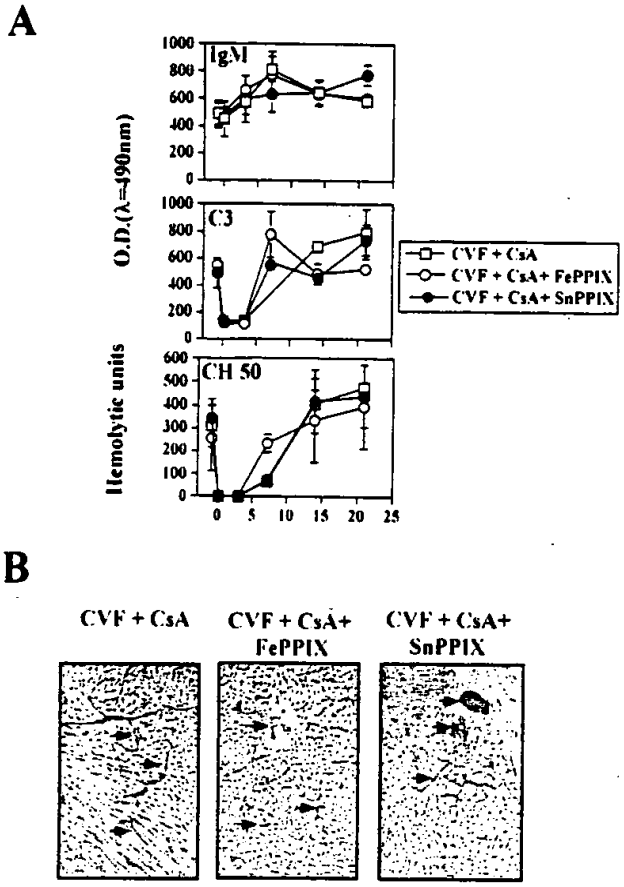

FIGURE 4. SnPPIX or FePPIX do not interfere with the generation of anti-graft Abs. Mouse hearts were transplanted into rats treated with CVF plus CsA. *A*, Serum level of anti-graft IgM Abs was evaluated by a cellular ELISA. Binding of rat C3 to mouse endothelial cells was evaluated by cellular ELISA. CH50 assay was evaluated by a standard hemolytic assay. Results shown are the mean ± SD (n = 3). *B*, Mouse hearts were transplanted into rats treated with CVF plus CsA. Hearts were harvested 3 days after transplantation and evaluated for the binding of rat IgM to the mouse vascular endothelium. Results shown are one representative section from three animals analyzed. Samples (magnification ×60) were counterstained with hematoxilin.

Table II. *Exogenous CO fully substitutes HO-1 in suppressing graft rejection*[a]

Treatment	Survival Time (days)
CVF + CsA + SnPPIX	3, 4, 5 (n = 2); 6 (n = 4); 7 (n = 2)
CVF + CsA + SnPPIX + CO	>50 (n = 6)

[a] Mouse hearts were transplanted into CVF plus CsA-treated rats. When indicated, graft recipients were treated with SnPPIX with or without exposure to CO, as described in *Materials and Methods*. Graft rejection observed in SnPPIX-treated rats was suppressed under exposure to exogenous CO ($p < 0.0001$ as compared to recipients treated with CVF plus CsA plus SnPPIX). Statistical analyzes were carried out using Fisher's exact test.

livers was also up-regulated after transplantation in a manner that mimicked that of the transplanted hearts (data not shown). However, this was not the case for the recipient's heart, in which HO activity was not up-regulated following transplantation (Fig. 2).

In grafts transplanted into SnPPIX-treated rats, there was progressive myocardial infarction, which became apparent as early as 2 days after transplantation (Fig. 3). This was not observed in grafts transplanted into control rats treated with FePPIX (Fig. 3).

We have previously shown that rats that receive a mouse cardiac graft under CVF plus CsA treatment generate anti-mouse Abs that are exclusively of the IgM isotype (4). Additional treatment with SnPPIX or FePPIX did not influence this Ab response (Fig. 4). Generation of antigraft Abs was correlated by complement activation, as demonstrated by C3 deposition on mouse endothelial cells (Fig. 4). Neither SnPPIX or FePPIX treatment influenced C3 deposition on mouse endothelial cells (Fig. 4).

Exogenous CO fully substitutes HO-1 enzymatic activity in suppressing acute vascular rejection

All mouse hearts transplanted into rats treated with SnPPIX and exposed to CO (400 ppm; 0.04%) survived long term (Table II). The dose of CO used (400–500 ppm) corresponds to approximately one-twentieth of the lethal dose (data not shown). Rats and mice exposed to CO did not exhibit untoward reactions. CO exposure was discontinued 14 (n = 3) or 16 (n = 3) days after transplantation without influencing graft survival, i.e., grafts continued to function for >50 days (Table II).

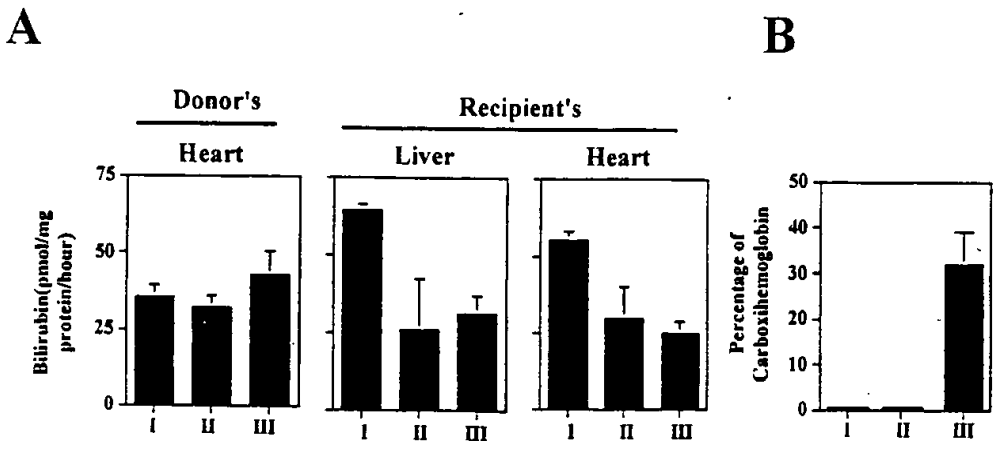

FIGURE 5. Exogenous CO does not affect the ability of SnPPIX to suppress HO-1 activity. *A*, Mouse hearts were transplanted into rats treated with CVF plus CsA plus SnPPIX (II) or SNPPIX and CO (III). Total HO activity was measured in donor's and recipient's hearts as well as in recipient's livers 2 days after transplantation. HO activity in different specimens was compared with basal HO activity in normal mouse hearts (I), rat hearts (I), or livers (I), according to the sample analyzed. Results shown are the mean ± SD of three animals analyzed for each treatment. Statistical analyses were conducted by using unpaired Welsh t test. *B*, The same animals were analyzed for carboxyhemoglobin content 2 days after transplantation. Results shown are the mean ± SD (n = 3).

Table III. *Morphometric analyzes*[a]

Treatment	Right Ventricle	Left Ventricle
CVF + CsA	4.5 ± 4.9	0.7 ± 2.1
CVF + CsA + FePPIX	12.2 ± 9.5	0.7 ± 1.3
CVF + CsA + SnPPIX	26.1 ± 12.7*	37.6 ± 15.5*
CVF + CsA + SnPPIX + CO	8.4 ± 5.3	1.8 ± 3.4

[a] Mouse hearts were transplanted into (n = 3 per group) CVF plus CsA-treated rats. When indicated graft recipients were treated with FePPIX and SnPPIX and exposed to CO, as described in *Materials and Methods*. Results are shown as percentage of infarcted area as described in *Materials and Methods*. Statistical analyzes were carried out using ANOVA test. An asterisk indicates significant difference as compared to all other treatments.

To determine whether exogenous CO interfered with inhibition of HO-1 enzymatic activity by SnPPIX, which could account for the ability of CO to suppress graft rejection, we tested whether or not CO affected HO enzymatic activity in hearts transplanted into SnPPIX-treated rats. As shown in Fig. 5, this was not the case. Total HO enzymatic activity in hearts transplanted under SnPPIX treatment (32.37 ± 7.23 pmol/mg/h) was not significantly different from that of hearts transplanted into rats treated with SnPPIX and exposed to CO (43.6 ± 7.57 pmol/mg/h; $p = 0.1095$; Fig. 5). Similar results were obtained in the recipient's livers and hearts (Fig. 5).

We have hypothesized that exogenous CO may substitute HO-1 activity in preventing graft rejection. Achieving such a result relied on the assumption that sufficient level of exogenous CO could be "loaded" by inhalation into RBC and then delivered through the circulation into the graft at an adequate concentration. Our assumption was that under inhibition of HO-1 activity by SnPPIX, exogenous CO would mimic the effect of endogenous CO that is produced when HO-1 enzymatic activity is not impaired. Exposure of the recipient to 400 ppm of exogenous CO increased carboxylhemoglobin from 0.5–1.5% to 32.1 ± 6.9% (Fig. 5). The fact that the transplanted hearts survived when exposed to CO, even under these suppressive effects of SnPPIX, shows that this level of CO was sufficient to adequately "charge" RBC, deliver CO into the graft, and suppress graft rejection (Fig. 5).

We then asked whether exogenous CO suppressed the development of myocardial infarction that characterizes graft rejection in SnPPIX-treated rats (Fig. 3). To test this hypothesis, grafts were harvested 3 days after transplantation and quantified for the percentage of infarcted area. Hearts transplanted into untreated rats showed nearly complete transmural infarction of the right ventricle (87.1 ± 4.9% of the right ventricle area) with extensive endomyocardial and transmural infarction of the left ventricle (32.0 ± 6.7% of the left ventricle area; data not shown). Infarctions showed nonviable eosinophic myocardium lacking nuclei with interstitial hemorrhage, edema, and neutrophils. Left ventricle infarctions were always endomyocardial with transmural extension depending on the degree of infarction, and those in the right ventricle were more diffuse in origin. The percentage of infarcted area in both ventricles generally increased from the apex to the base of the heart. Hearts transplanted into CVF plus CsA-treated rats showed only small, diffuse, nontransmural areas of infarction in the right (4.5 ± 4.9%) but not in the left (0.7 ± 2.1%) ventricle (Table III). Hearts transplanted into CVF plus CsA plus FePPIX-treated rats showed small diffuse areas of infarction in the right (12.2 ± 9.5%) but not in the left (0.7 ± 1.3%) ventricle (Table III). These hearts were indistinguishable from those transplanted into CVF plus CsA-treated rats without FePPIX treatment (Fig. 6). Hearts transplanted into CVF plus CsA plus SnPPIX-treated rats showed significant transmural right ventricular infarctions (26.1 ± 12.7%) with extensive endomyocardial and transmural left ventricular infarctions (37.6 ± 15.5%) (Table III) in a pattern that was indistinguishable from that of hearts transplanted into untreated rats (Fig. 6). These lesions were specific to the transplanted heart. The recipients' native hearts did not develop any infarction. The percentage of infarcted area in hearts transplanted into SnPPIX-treated rats was significantly higher ($p < 0.001$) as compared with that of hearts transplanted into rats treated with CVF plus CsA with or without FePPIX treatment (Table III). Hearts transplanted into SnPPIX-treated rats that received exogenous CO showed very little infarction of the right (8.4 ± 5.3%) and left (1.8 ± 3.4%) ventricles (Table III), with patterns that were similar to those of hearts transplanted into CVF plus CsA-treated rats with or without FePPIX treatment (Fig. 6). The percentage of infarcted area in hearts transplanted into SnPPIX-treated rats that received exogenous CO was not significantly different from that of hearts transplanted into CVF plus CsA-treated rats with or without FePPIX treatment. However, the percentage of infarcted area in these hearts was significantly different ($p < 0.001$) from that of hearts transplanted under the same treatment but that did not receive exogenous CO.

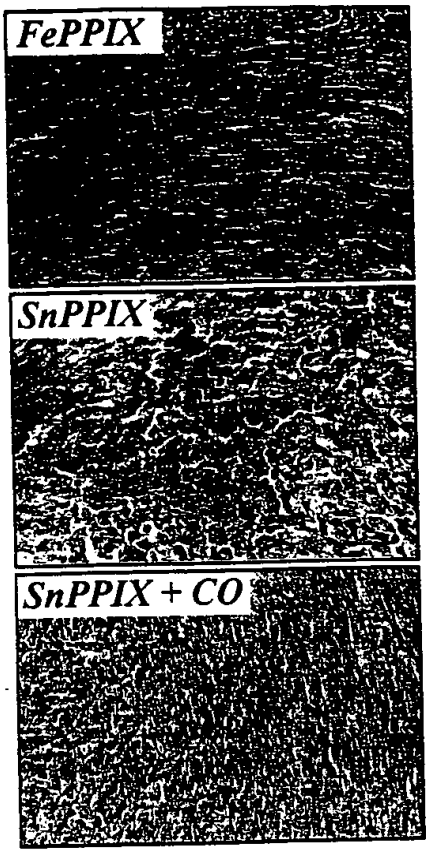

FIGURE 6. Exogenous CO suppresses myocardial infarction associated with the rejection of mouse hearts transplanted under SnPPIX treatment. Mouse cardiac grafts were harvested 3 days after transplantation (n = 3–6 per group) into CVF plus CsA-treated rats. When indicated, graft recipients were treated with FePPIX or SnPPIX and exposed to CO (250–400 ppm). Sections were stained with H&E. A representative field (magnification ×200) in the septal wall is illustrated for each treatment. Similar results were observed for left ventricular wall sections (data not shown).

Exogenous CO suppresses vascular thrombosis and monocyte/Mϕ infiltration that characterize acute vascular rejection

Mouse hearts transplanted into CVF plus CsA-treated rats with or without FePPIX treatment showed extensive intravascular deposition of rat IgM and C1q (Fig. 7) but no detectable IgG, C3, or C5b-9 (data not shown). HO-2, HO-1, and ferritin were detected in

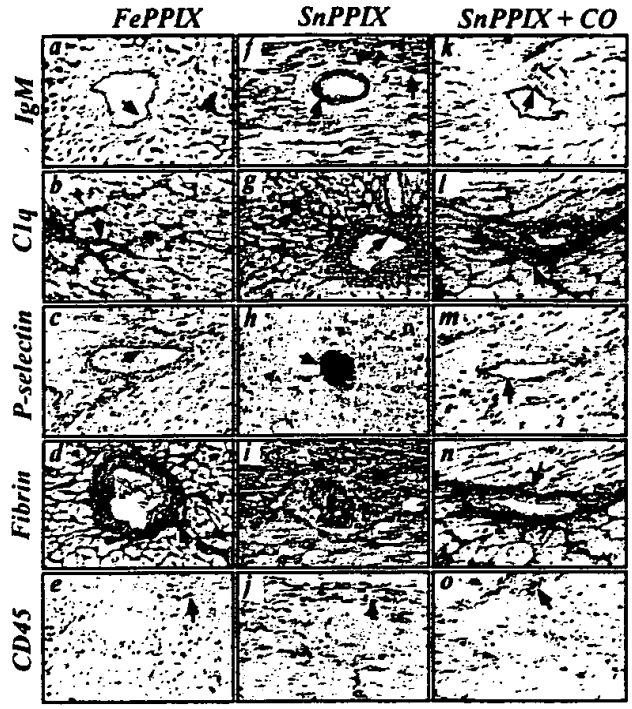

FIGURE 7. Exogenous CO suppresses vascular thrombosis and leukocyte infiltration associated with the rejection of mouse hearts transplanted under SnPPIX treatment. Mouse hearts were transplanted into CVF plus CsA-treated rats. When indicated, SnPPIX or FePPIX was administered and graft recipients were exposed to CO (250–400 ppm). Grafts were harvested 3 days after transplantation ($n = 3$ per group) and stained for rat IgM (*a*, *f*, and *k*), rat and mouse complement C1q (*b*, *g*, and *l*), rat and mouse P-selectin (*c*, *h*, and *m*), rat and mouse fibrin/fibrinogen (*d*, *i*, and *n*), and rat CD45 expressing leukocytes (*e*, *j*, and *o*). All sections are −600 magnifications counterstained with hematoxylin. Positive stainings are indicated by black arrows. Notice similar levels of rat IgM vascular deposition and rat C1q activation in hearts transplanted into control FePPIX- (*a* and *b*), SnPPIX- (*f* and *g*), or SnPPIX plus exogenous CO-treated (*k* and *l*) rats. P-selectin expression associated with platelet aggregation was observed in hearts transplanted into SnPPIX-treated rats (*h*) but not in hearts transplanted into control FePPIX-treated rats (*c*) or into rats treated with SnPPIX and exposed to CO from the time of transplantation (*m*). Fibrin/fibrinogen were detected lining the vasculature of all grafts, whereas intravascular fibrin deposition was detected only in hearts transplanted into SnPPIX-treated rats (*i*) but not in hearts transplanted into control FePPIX-treated rats (*d*) or into rats treated with SnPPIX and exposed to CO after transplantation (*n*). Extensive infiltration by rat CD45 leukocytes was observed in hearts transplanted into SnPPIX-treated rats (*j*), whereas minimal infiltration was observed in hearts transplanted into control FePPIX-treated rats (*e*) or into rats treated with SnPPIX and exposed to CO after transplantation (*o*).

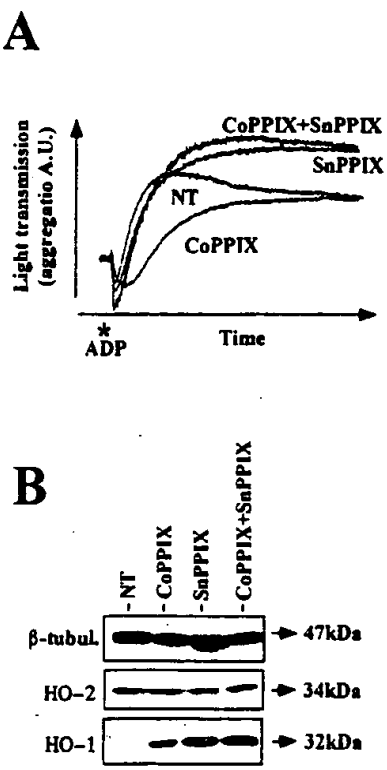

FIGURE 8. Up-regulation of HO-1 in endothelial cells inhibits platelet activation. Mouse 2F-2B endothelial cells were left untreated (NT) or were treated with CoPPIX (50 μM, 16 h) to up-regulate HO-1 activity, SnPPIX to suppress HO-1 activity (50 μM, 16 h), or CoPPIX (50 μM, 12 h) plus SnPPIX (50 μM, 4 h) to control for the specificity of CoPPIX in up-regulating HO-1 activity. *A*, Rat platelets were isolated, overlaid onto the mouse endothelial cells for 5 min, and tested for aggregation after stimulation with 2 μM ADP. *B*, HO-1 and HO-2 protein expression in untreated (NT), CoPPIX-, SnPPIX-, or CoPPIX plus SnPPIX-treated mouse endothelial cells was detected by Western blot. Results were normalized to the expression of β-tubulin. Notice that both CoPPIX and SnPPIX up-regulate HO-1 protein expression on mouse endothelial cells. However, SnPPIX inhibits HO-1 enzymatic activity whereas CoPPIX does not (38).

graft endothelial and smooth muscle cells as well as in cardiac myocytes (data not shown). There was only minimal vascular thrombosis or infiltration by host leukocytes usually associated with focal areas of infarction (Fig. 7). There was low but detectable P-selectin expression on the vascular endothelium (Fig. 7).

Hearts transplanted into CVF plus CsA-treated rats, under inhibition of HO-1 activity by SnPPIX, showed similar levels of intravascular deposition of IgM and C1q as compared with control FePPIX-treated rats (Fig. 7) and no detectable IgG, C3, or C5b-9 (data not shown). There was widespread vascular thrombosis of large coronary vessels associated with P-selectin-expressing platelet aggregates (Fig. 7) and intravascular fibrin (Fig. 7). Thrombi were consistently observed in large coronary vessels at the base of the heart. There were no detectable P-selectin-expressing platelet aggregates in the microvasculature (data not shown). There was extensive graft infiltration by host neutrophils as well as by $CD45^+$ leukocytes (Fig. 7) expressing the monocyte/Mϕ marker CD68/ED-1 and MHC class II Ags (data not shown). Infiltrating monocyte/Mϕ were found near arterioles and scattered throughout the myocardium, associated with areas of infarction (Fig. 7).

Hearts transplanted into SnPPIX-treated rats that were exposed to CO were essentially indistinguishable from those transplanted into rats treated with CVF plus CsA with or without FePPIX (Fig. 7). These hearts showed similar level of IgM and C1q vascular deposition as compared with hearts transplanted into recipients treated with SnPPIX but not exposed to CO (Fig. 7). Under CO exposure, there were no signs of vascular thrombosis as revealed by the lack of detectable P-selectin-expressing platelet aggregates or intravascular fibrin (Fig. 7). P-selectin was detected on the graft vascular endothelium (Fig. 7). There was some level of monocyte/Mϕ infiltration associated with small focal areas of infarction (Fig. 7).

Up-regulation of HO-1 in endothelial cells inhibits platelet aggregation

Given the absence of platelet aggregation in grafts transplanted into rats exposed to CO (Fig. 7), we questioned whether expression

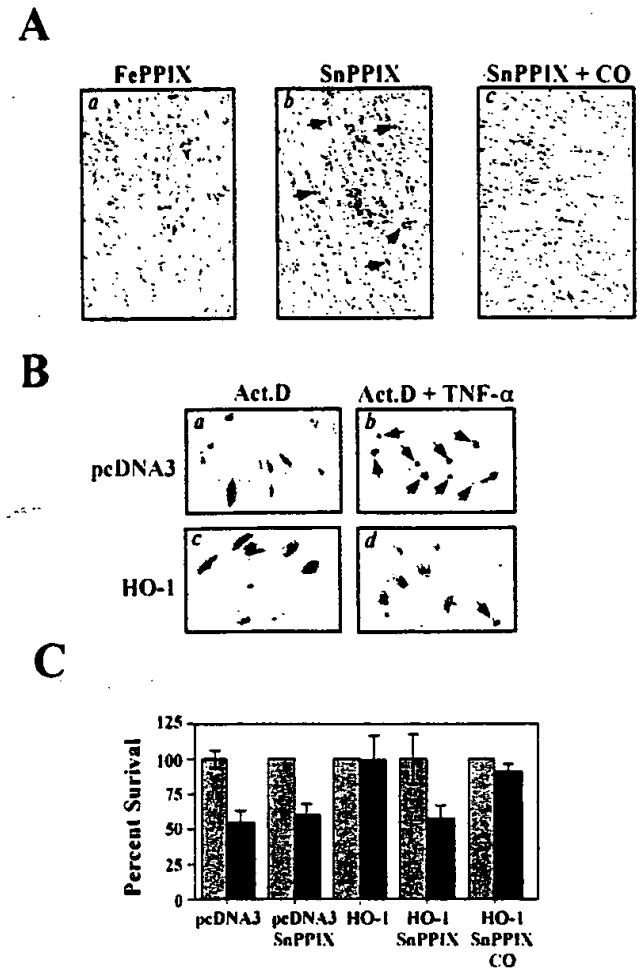

FIGURE 9. CO suppresses endothelial cell apoptosis. *A*, Mouse hearts where transplanted into rats treated with, CVF, CsA, and FePPIX, (*a*), CVF, CsA, and SnPPIX (*b*) or, CVF, CsA, SnPPIX, and CO (*c*). Hearts were harvested 3 days after transplantation and analyzed by using a peroxidase-based TUNEL technique. All panels are ×300 magnification from cryostat sections counterstained with hematoxylin. One representative sample from three animals is shown. *B*, Mouse 2F-2B endothelial cells were transiently cotransfected with β-galactosidase with or without HO-1. Transfected cells were stained with X-Gal and the number of "blue cells" retaining a normal morphology was counted. Notice rounded-shaped apoptotic cells (arrows) in samples treated with TNF-α plus Act.D. *C*, Mouse 2F-2B endothelial cells were transiently cotransfected with β-galactosidase with or without HO-1, as in *A*. Transfection with the pcDNA3 vector was used as a control. Gray histograms represent cells treated with Act.D alone and black histograms represent cells treated with Act.D plus TNF-α. When indicated, endothelial cells were treated with SnPPIX (50 μM) and exposed to exogenous CO (10,000 ppm). Results shown are mean ± SD of three independent experiments.

of HO-1 in endothelial cells would inhibit platelet aggregation in vitro. The hypothesis was that CO generated by endothelial cells expressing HO-1 might be sufficient to suppress platelet aggregation. To test this hypothesis, mouse endothelial cells were exposed to CoPPIX or SnPPIX to induce or suppress HO activity in these cells, respectively. Platelets were overlaid on the endothelial cells and tested for their ability to aggregate on stimulation by ADP (2 μM). Platelets overlaid on untreated endothelial cells aggregated normally when stimulated with ADP (Fig. 8). When platelets were exposed to endothelial cells pretreated with SnPPIX, platelet aggregation was enhanced as compared with platelets exposed to untreated endothelial cells (Fig. 8). This observation indicates that untreated endothelial cells have a basal level of HO activity presumably attributable to constitutive expression of HO-2 in these cells (Fig. 8). When platelets were exposed to endothelial cells pretreated with CoPPIX, platelet aggregation was significantly inhibited as compared with platelets exposed to untreated or SnPPIX-treated endothelial cells (Fig. 8). This inhibitory effect was suppressed when platelets were exposed to endothelial cells treated with both CoPPIX and SnPPIX (Fig. 8). Both CoPPIX and SnPPIX up-regulated the expression of HO-1 in cultured endothelial cells (Fig. 8). The differential effects of these protoporphyrins should be attributed to the ability of SnPPIX to act as a potent inhibitor of HO-1 enzymatic activity.

HO-1 generates CO that suppresses endothelial cell apoptosis

One of the main features that characterizes the rejection of mouse hearts transplanted into rats treated with SnPPIX is the widespread apoptosis of endothelial cells and cardiac myocytes (Fig. 9). Apoptosis did not occur in mouse hearts transplanted into rats treated with FePPIX (Fig. 9). Given the ability of HO-1 to suppress endothelial cell apoptosis in vitro (1, 28), we asked whether this cytoprotective effect was mediated via the generation of CO. Apoptosis did not occur in mouse hearts transplanted into rats treated with SnPPIX and exposed to CO, suggesting that this was the case (Fig. 9). To further test this hypothesis, we analyzed in vitro whether under inhibition of HO-1 activity by SnPPIX exogenous CO would suppress endothelial cells from undergoing TNF-α-mediated apoptosis. The data illustrated in Fig. 9 suggests that this is the case. Overexpression of HO-1 suppressed TNF-α-mediated endothelial cell apoptosis, such as it occurs in the presence of Act.D (Fig. 9; 1, 28). The antiapoptotic effect of HO-1 is mediated through its enzymatic activity because exposure of endothelial cells to SnPPIX blocked the antiapoptotic effect of HO-1 (Fig. 9). Under inhibition of HO-1 activity by SnPPIX, exogenous CO (10,000 ppm) suppressed TNF-α-mediated apoptosis, suggesting that HO-1 suppresses endothelial cell apoptosis via the generation of CO (Fig. 9).

Discussion

HO-1 is expressed by endothelial cells, smooth muscle cells, and myocytes of mouse cardiac grafts that survive long term in rats treated with CVF plus CsA (1, 3, 4, 6). Expression of HO-1 is essential to prevent graft rejection (1). The mechanism(s) by which HO-1 functions to prevent graft rejection are not well defined but presumably rely on the generation of one or several end products of heme degradation by this enzyme, i.e., bilirubin, free iron, and/or CO. We now provide evidence suggesting that this is the case and that this protective effect is attributable in large measure to the generation of CO. By using SnPPIX to suppress HO-1 enzymatic activity, we demonstrate that HO enzymatic activity is essential for the survival of mouse hearts that are transplanted into CVF plus CsA-treated rats (Table I and Fig. 3). Inhibiting HO-1 activity by SnPPIX led to graft rejection in a manner that was indistinguishable from that observed when HO-1-deficient (HO-$1^{-/-}$) mouse hearts are transplanted under the same immunosuppressive regimen, both with regard to the time of rejection (Table I and Fig. 3; Ref. 1) and to the pathogenesis of rejection (Figs. 3 and 7; Ref. 1). In the absence of HO-1 or under inhibition of HO activity by SnPPIX, graft rejection was characterized by widespread hemorrhagic infarction associated with vascular thrombosis and leukocyte infiltration (Figs. 3 and 7). These lesions did not occur in control recipients treated with FePPIX under the same immunosuppressive regimen (Figs. 3 and 7). This data suggests that HO-1 suppresses graft rejection by preventing thrombosis and

infarction that lead to this type of rejection, a finding consistent with those of others (32).

One possible explanation for the requirement of HO-1 to suppress graft rejection is that HO-1 enzymatic function is necessary to eliminate proinflammatory free heme as it accumulates through release of oxidized hemoglobin and myoglobin after transplantation. When HO-1 activity is suppressed, proinflammatory heme would accumulate and trigger endothelial cell activation and the expression of proinflammatory genes presumably involved in the pathogenesis of graft rejection (33). An alternative but not mutually exclusive explanation would be that HO enzymatic activity is needed to generate one or more of the end-products of heme catabolism, e.g. bilirubin, free iron that leads to ferritin expression, and/or CO. These antiinflammatory molecules would then abrogate the proinflammatory responses that lead to graft rejection. We favored the second hypothesis and tested directly whether the generation of CO would account for the protective effect of HO-1 in preventing graft rejection. To do so, mouse hearts were transplanted under inhibition of HO-1 activity by SnPPIX and exposed to exogenous CO. Exposure to CO fully suppressed graft rejection, allowing grafts to survive long term, despite the suppression of HO-1 activity by SnPPIX (Tables II and III). This data suggests that CO can fully substitute for the protective effect of HO-1 in this model. This data also suggests that the major mechanism by which HO-1 prevents graft rejection does not involve elimination of free heme but rather the generation of one of the end products of heme catabolism by HO-1, e.g., CO. We do not exclude that other end products of HO-1 activity, e.g., iron/ferritin and bilirubin, may contribute to prevent graft rejection as well. However, our present data shows that CO alone can fully substitute for HO-1.

It could be argued that the use of SnPPIX in these experiments is a rather nonspecific approach to demonstrate that HO-1 enzymatic activity is involved in preventing graft rejection. However, there are at least three observations that argue against this. First, under SnPPIX treatment, the pathogenesis of graft rejection (Table I and Fig. 3) is indistinguishable from that of hearts from HO-$1^{-/-}$ mice, transplanted under the same immunosuppressive regimen (1). Second, control treatment with FePPIX, a protoporphyrin that does not suppress HO-1 activity (Fig. 1) but that is otherwise very similar to SnPPIX, does not precipitate graft rejection (Table I). Third, one of the end products of HO-1 enzymatic activity, i.e., CO, can revert the ability of SnPPIX to precipitate graft rejection (Tables II and III). Taken together, these observations strongly support the notion that 1) SnPPIX precipitates graft rejection by suppressing HO-1 enzymatic activity and that 2) CO can revert the effect of SnPPIX by reconstituting the cytoprotective effect of endogenous CO that is generated when the action of HO-1 is not impaired.

The exact mechanism by which CO suppresses graft rejection remains to be elucidated. However, our present study provides some clues regarding these mechanism. One of the most significant effects of exogenous CO in our study was its ability to suppress platelet aggregation in the arterioles of the transplanted hearts (Fig. 7), a prominent feature observed during acute vascular rejection (9, 34). It is well established that CO has similar effects in vitro (35). Such effects of CO are also in keeping with our present data showing that expression of HO-1 in mouse endothelial cells suppressed platelet aggregation in vitro (Fig. 8), a finding similar to those reported by others using smooth muscle cells (24).

Although apoptosis of endothelial cells and cardiac myocytes is not a prominent feature associated with the rejection of mouse-to-rat cardiac transplants (1), widespread apoptosis occurs when these grafts cannot express HO-1 (e.g., grafts from HO-$1^{-/-}$ mice; Ref. 1) or when HO-1 enzymatic activity is inhibited by SnPPIX (Fig. 9). These data suggests that expression of HO-1 in vivo suppresses apoptosis. This notion is further supported by the fact that HO-1 can suppress endothelial cell apoptosis in vitro (1, 28). Given the proinflammatory effects associated with endothelial cell apoptosis, the antiapoptotic effect of HO-1 is likely to contribute to suppress graft rejection.

Under inhibition of HO-1 activity, exogenous CO can suppress endothelial cell apoptosis in vivo (Fig. 9), suggesting that the antiapoptotic effect of HO-1 is mediated through the generation of CO (Fig. 7). This is further supported by the finding that in the absence of HO-1 activity, exogenous CO suppresses endothelial cell apoptosis in vitro (Fig. 9). Given the above, we suggest that the antiapoptotic effect of CO is likely to contribute to the overall protective effect of HO-1 in preventing graft rejection.

CO may have additional effects that could contribute to prevent graft rejection. This include the ability of CO to promote vasodilatation (36) by the induction of smooth muscle cell relaxation (26, 37). Vasodilatation may contribute to prevent vascular thrombosis, which would be important in suppressing graft rejection. An additional antiinflammatory property of CO is its ability to suppress the expression of proinflammatory genes associated with the activation of monocyte/Mϕ (27). This is illustrated by the observation that CO suppresses TNF-α production while inducing the production of the anti-inflammatory cytokine IL-10 in activated Mϕ (27). In light of this observation, it is tempting to speculate that endothelial cells that express high levels of HO-1 and generate CO may modulate the activation of graft-infiltrating Mϕ in a manner that may contribute to suppress graft rejection.

In conclusion, our data suggests that the generation of CO by the graft vasculature, through the expression of the protective gene HO-1, plays a critical role in promoting the survival of cardiac transplants. We suggest that CO acts as an anti-inflammatory molecule that induces vasodilatation while suppressing platelet aggregation and proinflammatory monocyte/Mϕ activation. In addition, CO has antiapoptotic effects over the graft endothelium that may contribute to suppress graft rejection as well. Potential therapeutic applications of these findings include the possibility to overexpress HO-1 in endothelial cells of a graft to generate CO at the time of transplantation. Although our studies have used a xenotransplantation as a model of vascular inflammatory injury, it seems likely that these effects of CO might be used therapeutically in other inflammatory processes.

References

1. Soares, M. P., Y. Lin, J. Anrather, E. Csizmadia, K. Takigami, K. Sato, S. T. Grey, R. B. Colvin, A. M. Choi, K. D. Poss, and F. H. Bach. 1998. Expression of heme oxygenase-1 (HO-1) can determine cardiac xenograft survival. *Nat. Med. 4:1073.*
2. Bach, F. H., C. Ferran, M. Soares, C. J. Wrighton, J. Anrather, H. Winkler, S. C. Robson, and W. W. Hancock. 1997. Modification of vascular responses in xenotransplantation: inflammation and apoptosis. *Nat. Med. 3:944.*
3. Bach, F. H., C. Ferran, P. Hechenleitner, W. Mark, N. Koyamada, T. Miyatake, H. Winkler, A. Badrichani, D. Candinas, and W. W. Hancock. 1997. Accommodation of vascularized xenografts: expression of "protective genes" by donor endothelial cells in a host Th2 cytokine environment. *Nat. Med. 3:196.*
4. Koyamada, N., T. Miyatake, D. Candinas, W. Mark, P. Hechenleitner, W. W. Hancock, M. P. Soares, and F. H. Bach. 1998. Transient complement inhibition plus T-cell immunosuppression induces long-term survival of mouse-to-rat cardiac xenografts. *Transplantation 65:1210.*
5. Soares, M. P., Y. Lin, K. Sato, K. M. Stuhlmeier, and F. H. Bach. 1999. Accommodation. *Immunol. Today 20:434.*
6. Soares, M. P., Y. Lin, K. Sato, K. Takigami, J. Anrather, C. Ferran, S. C. Robson, and F. H. Bach. 1999. Pathogenesis of and potential therapies for delayed xenograft rejection. *Curr. Opin: Organ Trans. 4:80.*
7. Parker, W., S. Saadi, S. S. Lin, Z. E. Holzknecht, M. Bustos, and J. L. Platt. 1996. Transplantation of discordant xenografts: a challenge revisited. *Immunol. Today 17:373.*
8. Miyatake, T., K. Sato, K. Takigami, N. Koyamada, W. W. Hancock, H. Bazin, D. Latinne, F. H. Bach, and M. P. Soares. 1998. Complement-fixing elicited

antibodies are a major component in the pathogenesis of xenograft rejection. *J. Immunol. 160:4114.*
9. Bach, F. H., S. C. Robson, C. Ferran, H. Winkler, M. T. Millan, K. M. Stuhlmeier, B. Vanhove, M. L. Blakely, van, der, Werf, Wj, E. Hofer, et al. 1994. Endothelial cell activation and thromboregulation during xenograft rejection. *Immunol. Rev. 141:5.*
10. Bach, F. H., M. A. Turman, G. M. Vercellotti, J. L. Platt, and A. P. Dalmasso. 1991. Accommodation: a working paradigm for progressing toward clinical discordant xenografting. *Trans. Proc. 23:205.*
11. Lin, Y., M. P. Soares, K. Sato, K. Takigami, E. Csizmadia, N. Smith, and F. H. Bach. 1999. Accommodated xenografts survive in the presence of anti-donor antibodies and complement that precipitate rejection of naive xenografts. *J. Immunol. 163:2850.*
12. Soares, M. P., S. Brouard, R. N. Smith, L. Otterbein, A. M. Choi, and F. H. Bach. 2000. Expression of heme oxygenase-1 by endothelial cells: a protective response to injury in transplantation. *Emerging Ther. Targets 4:11.*
13. Choi, A. M., and J. Alam. 1996. Heme oxygenase-1: function, regulation, and implication of a novel stress-inducible protein in oxidant-induced lung injury. *Am. J. Respir. Cell Mol. Biol. 15:9.*
14. Willis, D. 1999. Overview of HO-1 in inflammatory pathologies. In *Inducible Enzymes in the Inflammatory Response.* D. A. Willoughby and A. Tomlinson, eds. Birkhauser, Basel, p. 55.
15. Poss, K. D., and S. Tonegawa. 1997. Heme oxygenase 1 is required for mammalian iron reutilization. *Proc. Natl. Acad. Sci. USA 94:10919.*
16. Poss, K. D., and S. Tonegawa. 1997. Reduced stress defense in heme oxygenase 1-deficient cells. *Proc. Natl. Acad. Sci. USA 94:10925.*
17. Otterbein, L. E., J. K. Kolls, L. L. Mantell, J. L. Cook, J. Alam, and A. M. Choi. 1999. Exogenous administration of heme oxygenase-1 by gene transfer provides protection against hyperoxia-induced lung injury. *J. Clin. Invest. 103:1047.*
18. Willis, D., A. R. Moore, R. Frederick, and D. A. Willoughby. 1996. Heme oxygenase: a novel target for the modulation of the inflammatory response. *Nat. Med. 2:87.*
19. Abraham, N. G., Y. Lavrovsky, M. L. Schwartzman, R. A. Stoltz, R. D. Levere, M. E. Gerritsen, S. Shibahara, and A. Kappas. 1995. Transfection of the human heme oxygenase gene into rabbit coronary microvessel endothelial cells: protective effect against heme and hemoglobin toxicity. *Proc. Natl. Acad. Sci. USA 92:6798.*
20. Ferris, C., S. Jaffrey, A. Sawa, M. Takahashi, S. Brady, R. Barrow, S. Tysoe, H. Wolosker, D. Baranano, S. Dore, K. Poss, and S. H. Snyder. 1999. Haem oxygenase-1 prevents cell death by regulating cellular iron. *Nat. Cell Biol. 1:152.*
21. Petrache, I., L. E. Otterbein, J. Alam, G. W. Wiegand, and A. M. Choi. 2000. Heme oxygenase-1 inhibits TNF-α-induced apoptosis in cultured fibroblast. *Am. J. Physiol. Lung Cell. Mol. Physiol 278:312.*
22. Yachie, A., Y. Niida, T. Wada, N. Igarashi, H. Kaneda, T. Toma, K. Ohta, Y. Kasahara, and S. Koizumi. 1999. Oxidative stress causes enhanced endothelial cell injury in human heme oxygenase-1 deficiency. *J. Clin. Invest. 103:129.*
23. Otterbein, L. E., L. L. Mantell, and A. M. Choi. 1999. Carbon monoxide provides protection against hyperoxic lung injury. *Am. J. Physiol. 276:L688.*
24. Wagner, C. T., W. Durante, N. Christodoulides, J. D. Hellums, and A. I. Schafer. 1997. Hemodynamic forces induce the expression of heme oxygenase in cultured vascular smooth muscle cells. *J. Clin. Invest. 100:589.*
25. Morita, T., and S. Kourembanas. 1995. Endothelial cell expression of vasoconstrictors and growth factors is regulated by smooth muscle cell-derived carbon monoxide. *J. Clin. Invest. 96:2676.*
26. Morita, T., M. A. Perrella, M. E. Lee, and S. Kourembanas. 1995. Smooth muscle cell-derived carbon monoxide is a regulator of vascular cGMP. *Proc. Natl. Acad. Sci. USA 92:1475.*
27. Otterbein, L. E., F. H. Bach, J. Alam, M. P. Soares, H. L. Tao, M. Wysk, R. Davis, R. Flavell, and A. M. K. Choi. 2000. Carbon monoxide mediates anti-inflammatory effects via the mitogen activated protein kinase pathway. *Nat. Med. 6:422.*
28. Brouard, S., L. E. Otterbein, J. Anrather, E. Tobiasch, F. H. Bach, A. M. K. Choi, and M. P. Soares. 2000. Carbon Monoxide genrated by heme oxygenase-1 suppresses endothelial cell apoptosis. *J. Exp. Med. 192:1015.*
29. Hancock, W. W., R. Buelow, M. H. Sayegh, and L. A. Turka. 1998. Antibody-induced transplant arteriosclerosis is prevented by graft expression of anti-oxidant and anti-apoptotic genes. *Nat. Med. 4:1392.*
30. Brouard, S., M. C. Cuturi, P. Pignon, R. Buelow, P. Loth, A. Moreau, and J. P. Soulillou. 1999. Prolongation of heart xenograft survival in a hamster-to-rat model after therapy with a rationally designed immunosuppressive peptide. *Transplantation 67:1614.*
31. Kaczmarek, E., K. Koziak, J. Sevigny, J. B. Siegel, J. Anrather, A. R. Beaudoin, F. H. Bach, and S. C. Robson. 1996. Identification and characterization of CD39/vascular ATP diphosphohydrolase. *J. Biol. Chem. 271:33116.*
32. Yet, S. F., M. A. Perrella, M. D. Layne, C. M. Hsieh, K. Maemura, L. Kobzik, P. Wiesel, H. Christou, S. Kourembanas, and M. E. Lee. 1999. Hypoxia induces severe right ventricular dilatation and infarction in heme oxygenase-1 null mice. *J. Clin. Invest. 103:R23.*
33. Wagener, E. Feldman, T. de Witte, and N. G. Abraham. 1997. Heme induces the expression of adhesion molecules ICAM-1, VCAM-1, and E selectin in vascular endothelial cells. *Proc. Soc. Exp. Biol. Med. 216:456.*
34. Bach, F. H., H. Winkler, C. Ferran, W. W. Hancock, and S. C. Robson. 1996. Delayed xenograft rejection. *Immunol. Today 17:379.*
35. Brune, B., and V. Ullrich. 1987. Inhibition of platelet aggregation by carbon monoxide is mediated by activation of guanylate cyclase. *Mol. Pharmacol. 32:497.*
36. Motterlini, R., A. Gonzales, R. Foresti, J. E. Clark, C. J. Green, and R. M. Winslow. 1998. Heme oxygenase-1-derived carbon monoxide contributes to the suppression of acute hypertensive responses in vivo. *Circ. Res. 83:568.*
37. Yet, S. F., A. Pellacani, C. Patterson, L. Tan, S. C. Folta, L. Foster, W. S. Lee, C. M. Hsieh, and M. A. Perrella. 1997. Induction of heme oxygenase-1 expression in vascular smooth muscle cells: a link to endotoxic shock. *J. Biol. Chem. 272:4295.*
38. Smith, A., J. Alam, P. V. Escriba, and W. T. Morgan. 1993. Regulation of heme oxygenase and metallothionein gene expression by the heme analogs, cobalt-, and tin-protoporphyrin. *J. Biol. Chem. 268:7365.*